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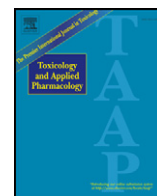
Galantamine is a novel post-exposure therapeutic against lethal VX challenge ☆

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ABSTRACT

The ability of galantamine hydrobromide (GAL HBr) treatment to antagonize O-ethyl-S-(2-diisopropylaminoethyl) methylphosphonothiolate (VX)-induced lethality, impairment of muscle tension, and electroencephalographic (EEG) changes was assessed in guinea pigs. Guinea pigs were challenged with 16.8 μg/kg VX (2LD50). One min after challenge, animals were administered 0.5 mg/kg atropine sulfate (ATR) and 25 mg/kg pyridine-2-aldoxime methochloride (2-PAM). In addition, guinea pigs were given 0, 1, 2, 4, 8 or 10 mg/kg GAL as a post-exposure treatment immediately prior to ATR and 2-PAM. Animals were either monitored for 24-h survival, scheduled for electroencephalography (EEG) recording, or euthanized 60 min later for measurement of indirectly-elicited muscle tension in the hemidiaphragm. Post-exposure GAL therapy produced a dose-dependent increase in survival from lethal VX challenge. Optimal clinical benefits were observed in the presence of 10 mg/kg GAL, which led to 100% survival of VX-challenged guinea pigs. Based on muscle physiology studies, GAL post-exposure treatment protected the guinea pig diaphragm, the major effector muscle of respiration, from fatigue, tetanic fade, and muscular paralysis. Protection against the paralyzing effects of VX was dose-dependent. In EEG studies, GAL did not alter seizure onset for all doses tested. At the highest dose tested (10 mg/kg), GAL decreased seizure duration when administered as a post-exposure treatment 1 min after VX. GAL also reduced the high correlation associated between seizure activity and lethality after 2LD50 VX challenge. GAL may have additional benefits both centrally and peripherally that are unrelated to its established mechanism as a reversible acetylcholinesterase inhibitor (AChEI).

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Introduction

Organophosphorus (OP) compounds have long been used as pesticides and chemical warfare agents (CWAs). Occupational exposure to OP pesticides afflicts millions of people and leads to hundreds of thousands of deaths every year throughout the world (reviewed in Sogorb et al., 2004). OPs used as weapons of mass destruction are far more toxic than those used as pesticides and are suitable for terrorist attacks such as those in Japan in 1994 and 1995 (Morita et al., 1995; Ohbu et al., 1997).

The acute toxicity of organophosphorus nerve agents is due principally to their irreversible inhibition of acetylcholinesterase (AChE), an enzyme present at all known cholinergic synapses (Taylor, 1996). AChE serves to limit the duration of the neurotransmitter acetylcholine (ACh) and thus to prevent its accumulation at synaptic junctions. Exposure to AChE inhibitors results in symptoms indicative of widespread overstimulation of ACh receptors. These symptoms include bronchoconstriction, increases in tracheobronchial secretion, lacrimation, muscle weakness, convulsion, loss of consciousness and death (Hoskins et al., 1986; Taylor, 1996). The standard U.S. military therapy for intoxication by anticholinesterase agents consists of administering ATR to antagonize excessive muscarinic stimulation and pyridine-2-aldoxime methochloride (2-PAM) to reactivate inhibited AChE. Oxime is a critical treatment strategy against agents like VX that undergo slow aging. For organophosphorus inhibitors that undergo rapid aging, such as soman, 2-PAM confers little benefit since AChE becomes refractory to reactivation within several minutes of exposure (Berman and Decker, 1986). In this case, a useful strategy might be to protect a critical pool of AChE from irreversible inhibition by pretreatment with the carbamate pyridostigmine bromide (PB) (Gordon et al., 1978; French et al., 1979).

While the soman nerve agent PB pretreatment (SNAPP) is effective in protecting against soman toxicity when followed by ATR and 2-PAM therapy, it has several disadvantages. While protection occurs at doses of PB that produce little or no overt symptoms of AChE inhibition immediately (Caldwell et al., 1989; Dawson, 1994; Marino et al., 1998), PB was shown to have long term side effects. A Congressional panel report, released November 17, 2008, concluded that the ingestion of PB pills, provided to warfighters during the Persian Gulf War to protect against nerve agent, was a major cause of Gulf War illness in addition to pesticides (RAC, 2008). Another obvious disadvantage of any pre-exposure treatment strategy is the added risk of ingesting or receiving a chemical compound and never encountering the threat against which it was designed to protect. PB is able to enhance survival from soman-induced lethality but cannot prevent seizures or other centrally-mediated toxic actions of nerve agent exposure. The highly lipid soluble nerve agents easily penetrate the blood brain barrier (BBB), irreversibly inhibit brain ChE, and cause seizures. Additional therapeutic treatment with benzodiazepine (BZD) anticonvulsants is required.

Neurodegeneration in the hippocampus, pyriform cortex, and amygdala is a hallmark of nonfatal OP exposure in animals. It probably contributes to the delayed cognitive effects observed in the survivors of the sarin subway attack in Japan (DiGiovanni, 1999). An ideal therapeutic candidate should be neuroprotective and demonstrate efficacy and safety. Galantamine (GAL), an alkaloid and reversible AChEI, is approved for the treatment of mild to moderate Alzheimer's disease (Corey-Bloom, 2003). A ball-and-stick representation of GAL is shown in Fig. 1 docking to the active site of two different acetylcholinesterase forms. GAL displays considerable advantages as a putative countermeasure against nerve agent. GAL is capable of crossing the blood brain barrier to penetrate the central nervous system (CNS). GAL is a neuroprotectant and prevents neurodegeneration (Pereira et al., 2002; Arias et al., 2004; Kihara et al., 2004), a classic sequelae of nerve agent intoxication. Finally, GAL demonstrates protection against soman- or sarin-induced lethality when combined with high doses of ATR (10 mg/kg) (Albuquerque et al., 2006).

A multidisciplinary approach was used to evaluate GAL as a next-generation chemical warfare nerve agent therapeutic and neuroprotectant in guinea pigs, when combined with an approved treatment regimen of ATR (0.5 mg/kg) and 2-PAM (25 mg/kg), against a lethal 2LD50 challenge of VX. This treatment regimen (ATR + 2-PAM) is approved by the FDA. The doses were carefully chosen to be analogous to the immediate aid that a nerve agent exposed warfighter would realistically receive in the field and are in agreement with consensus treatment doses recommended for biomedical research in the US Army. The lethal VX dose was selected such that conventional ATR and 2-PAM would not promote animal survival as a stand-alone treatment at the doses listed above. Guinea pigs were chosen as the most appropriate nonprimate animal model to test antidotal therapy effectiveness against lethal nerve agents (Maxwell et al., 1988) for several reasons. Small mammals are desirable for the proposed study because almost all carbamate protection studies against nerve agent toxicity have been carried out on small mammals. Guinea pigs are preferred since they are approximately 3-fold more sensitive to nerve agents than rats and mice (Dawson, 1994), due presumably to their low concentration of circulating carboxylesterase. High levels of blood carboxylesterase present in rats and mice complicate interpretation of protection studies by yielding apparently low protective ratios (Hilmas et al., 2006).

The objective of this study was to establish the doses of GAL, administered in the post-exposure treatment period, that enhance the 24-h survival of guinea pigs after exposure to the nerve agent VX, offer peripheral protection of diaphragmatic function from VX-induced impairment, and provide central protection from nerve agent induced seizures. Whole blood or RBC AChE activities have been used as surrogate markers to define the effective doses of PB, a peripherally-acting carbamate, and to assess intoxication and recovery from nerve agent exposure. Blood AChE is a convenient determinant and should reflect tissue levels following nerve agent exposure. While inhibition of blood AChE plays no role in nerve agent intoxication, it was desirable to demonstrate that protective doses of GAL can preserve function in a critical target such as the phrenic nerve-hemidiaphragm preparation through reversible inhibition of AChE.

Materials and methods

The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, Publication No. 85-
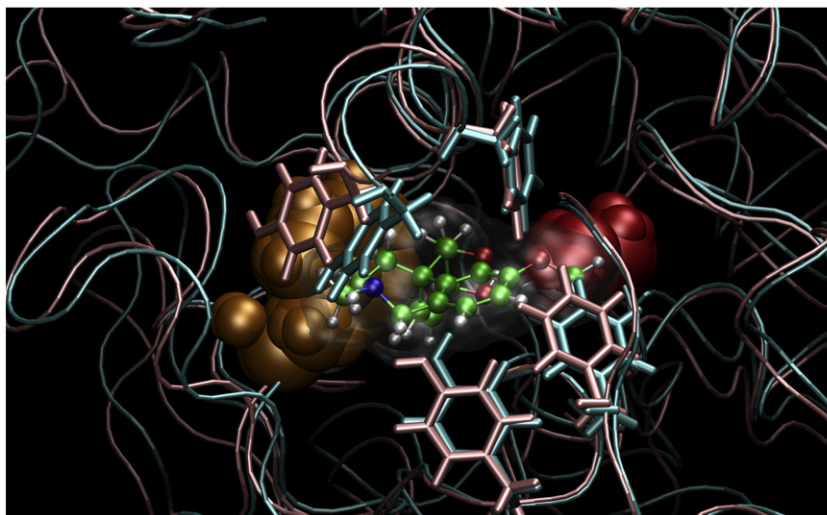
Fig. 1. Three-dimensional model of galantamine docking into human and *Torpedo californica* (Tc) forms of acetylcholinesterase (AChE). Human AChE is displayed in light blue and TcAChE in pink, except for the large, space-filling ball display. The orange ball region on the left-hand side (also known as CPK) is the active site tryptophan. The red ball display of AChE (on the right-hand side) is the active site serine. Galantamine, represented as a ball-and-stick model configuration, is colored by atom type and has a transparent solvent accessible surface, represented as a gray cloud, to demonstrate that it is filling a pocket. The docking was performed using Insight II software by manually aligning the backbones of the two AChE forms shown.

23, 1996) and the Animal Welfare Act of 1996 (P.L. 89-544), as amended. Male Hartley guinea pigs (300–350 g) from Charles River Laboratories (Wilmington, MA) were housed in accordance with an IACUC-approved protocol and provided commercial ration and acidified tap water *ad libitum*. Animal holding rooms were maintained at $70 \pm 2\,°F$ with $50 \pm 10\%$ relative humidity using at least 10 complete changes per hour of 100% conditioned fresh air. The guinea pigs were on a 12-h light/dark full-spectrum lighting cycle.

A schematic timeline of the experimental paradigm indicating time points for blood collection, agent challenge, post-exposure treatment therapy, and euthanization of animal is shown in Fig. 2. Guinea pigs were exposed to a 2LD50 nerve agent challenge of VX (16.8 μg/kg) via a midline, s.c. injection in the dorsal thoracic region. In this post-exposure treatment protocol, guinea pigs were randomly assigned to one of six different treatment groups of GAL (0, 1, 2, 4, 8, or 10 mg/kg; Tocris Bioscience, Ellisville, MO). All animals received a treatment dose of GAL, followed by ATR (0.5 mg/kg) and the oxime 2-PAM (25 mg/kg) (Sigma-Aldrich, St. Louis, MO), 1 min after VX exposure. ATR and 2-PAM were admixed in the same syringe and delivered i.m. as a single injection. The treatment regimen of ATR and 2-PAM is approved by the FDA, and the dose analogous to the immediate aid of three Mark I doses that a warfighter would receive in the field if he had severe nerve agent exposure. Animals were observed for clinical signs of toxicity over the following 4 h.

General procedures.   After receiving injections, guinea pigs were used immediately in one of 3 ways. Guinea pigs were scheduled for either (*i*) immediate clinical observations, toxic signs scoring, and determination of 24-h survival; (*ii*) ex vivo muscle physiology to assess skeletal muscle (diaphragm) recovery from nerve agent paralysis; or (*iii*) electroencephalography (EEG) to evaluate protection against overt and subclinical seizures. Blood was also collected by toe clip sampling from animals scheduled for muscle physiology; blood was utilized for determination of red blood cell (RBC) acetylcholinesterase (AChE) activity at the various time points shown in Fig. 2.

Ex vivo muscle physiology studies.   Guinea pigs were rendered unconscious in an isoflurane chamber and euthanized by decapitation after the animal was unresponsive to strong ear pinch. The dissection methods for removal of the phrenic nerve-diaphragm muscle preparation were similar to that described for rat and mouse diaphragms (Deshpande et al., 1997). The diaphragm and attached phrenic nerves were removed *en bloc* and placed in oxygenated Krebs–Ringer solution, equilibrated with a 95% O_2/5% CO_2 gas mixture at 37 °C. Each hemidiaphragm muscle was suspended under tension in a water-jacketed tissue bath (Radnoti) and continuously oxygenated. One end of the muscle was anchored to the chamber by a stainless steel hook, while the tendon was tied to a force-displacement transducer with 4–0 silk thread. The phrenic nerve was stimulated with 100 μs supramaximal pulses (5 V) using bipolar platinum electrodes. The resulting tensions were measured using Grass (Warwick, RI) isometric force transducers, recorded on a Gould chart recorder and analyzed off-line with pClamp electrophysiological software. The first ten twitch tensions (0.05 Hz frequency stimulation) were recorded, followed 2 min later by supramaximal stimulation of the nerve in the physiological relevant range (50–200 Hz) (Wright, 1954; Cohen, 1979) to generate tetanic force tensions.

Time course for inhibition of whole blood AChE.   Venous blood samples were taken by toe clip sampling from non-anesthetized guinea pigs, designated for muscle physiology, and collected into heparinized tubes at three time intervals (1B, 2B, 3B) listed in Fig. 2. The first blood sample (1B) was taken just prior to the first injection (VX nerve agent). An aliquot (100 μL) of blood was immediately removed and flash frozen at −80 °C. All flash frozen whole blood was used to determine blood AChE activity. This procedure was followed for the second blood sample (2B), taken 30 min later. The third blood sample (3B) was obtained upon euthanization (30 min after sample 2B and 60 min after sample 1B) following decapitation of the anesthetized animal. Guinea pigs were anesthetized with isoflurane using an induction chamber just prior to euthanasia.
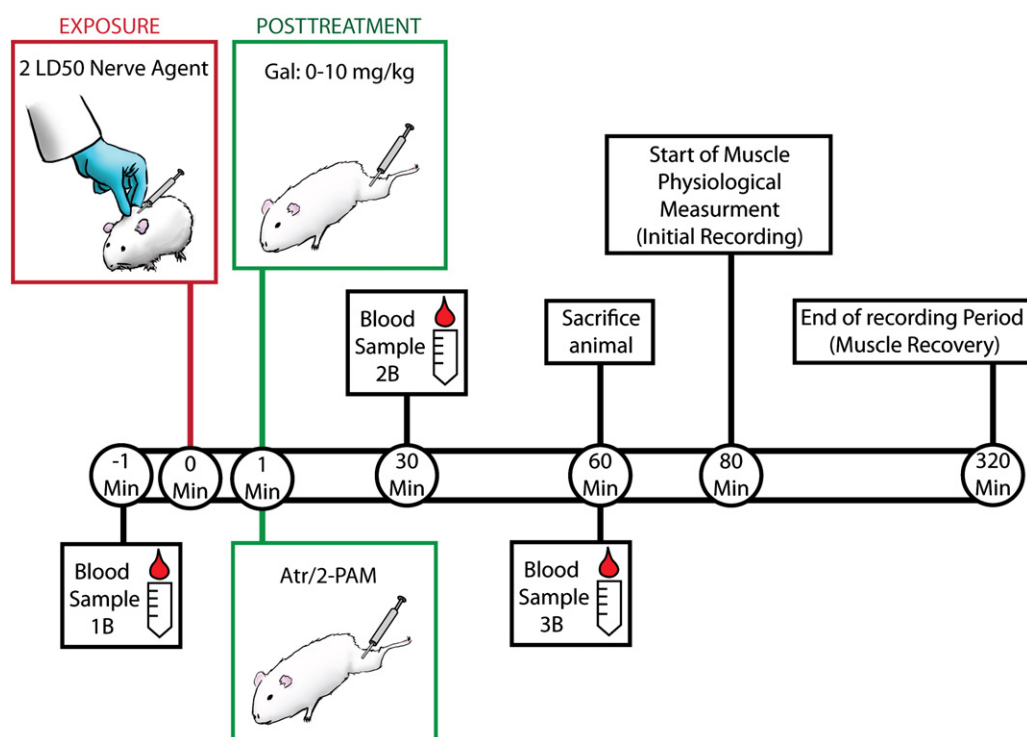

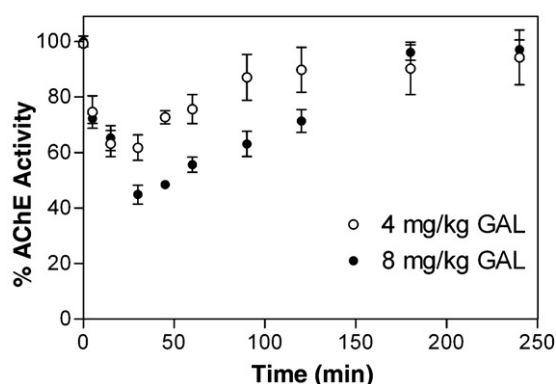Fig. 2. Schematic of treatment injection paradigm.

Fig. 3. Temporal relationship between GAL dose and blood AChE activity. The time course for inhibition of whole blood AChE in guinea pigs injected i.m. with 4 or 8 mg/kg GAL is shown. Whole blood was collected at various time points with respect to GAL injection and assayed for AChE activity. AChE activity (% activity of the control) was plotted as a function of time. Each data point and bars represent the mean ± SEM of data obtained from 4 animals. Control AChE activity is defined immediately before GAL injection. Percent AChE activity is calculated as the measured activity at a given time point divided by control AChE activity × 100%.

Measurement of AChE activity in whole blood. AChE activity was measured by the method described earlier (Feaster et al. 2000). Ten µL of blood was transferred to a standard 96 well microliter plate containing the chromophore 4,4′-dipyridyl disulfide and one of three substrates at 1 mM: acetylthiocholine, propionylthiocholine or butyrylthiocholine. Each condition was performed in triplicate. The reaction was monitored at room temperature for 4 min at 324 nm using a Molecular Device SpectraMax Plus spectrophotometer (Sunnyvale, CA). The data was subjected to least squares analysis, from which the sensitivity coefficients for AChE and BChE were extracted.

Surgery for EEG electrode placement. Guinea pigs were anesthetized with isoflurane and stereotaxically implanted with stainless steel cortical screw electrodes using standard rodent aseptic surgical techniques previously described (McDonough and Shih, 1993). Stainless steel cortical EEG screws were placed approximately 3.0 mm lateral from midline and equidistant between bregma and lambda. The screws in the skull were attached to a miniature connector via stainless steel wires, and the assembly was subsequently anchored to the skull with dental acrylic. The animals were allowed to recover for at least 5 days prior to experimentation.

Recording of EEG activity. Each guinea pig was placed in a single EEG recording chamber with clear front panels. Individual animal headpieces were connected to the EEG recording apparatus, and QND Software and amplifiers (NeuroData, Inc., Pasadena, CA) were used to record activity. During the 4-h recording period after nerve agent challenge, the time to seizure onset and seizure duration were the parameters evaluated.

Data collection. Analog data from the isometric force transducers were stored in a digital computer on-line using an analog-to-digital converter. The digitized waveforms were analyzed off-line to determine the peak amplitude and area under the curve (AUC) of twitch and tetanic tensions for each muscle contraction. A digital computer was also used to initiate electrical stimuli to the muscle via an analog stimulus isolator. Twitch and tetanic tension data were used to construct graphs of hemidiaphragm muscle recovery after nerve agent challenge.

Data analysis. Unless stated otherwise, all values are expressed as the mean ± SEM. Data were analyzed by a one-way analysis of variance followed by the Tukey–Kramer multiple comparison test (GraphPad InStat or Prism, GraphPad Software, San Diego, CA). For all tests, statistical significance is defined as $p < 0.05$. For survival studies, a Chi-square analysis followed by a Fisher's exact test was used to compare pairs of groups with respect to the percent survival.

Drugs and solutions. The Krebs–Ringer solution was bubbled with carbogen, a mixture of 95% O_2 and 5% CO_2, and had the following composition (mM): NaCl, 135; KCl, 5.0; $MgCl_2$, 1.0; $CaCl_2$, 2.0; $NaHCO_3$, 15; Na_2HPO_4, 1; and glucose, 11 (pH 7.3).

Results

Time course of GAL effects on AChE activity in whole blood

To determine the appropriate interval between GAL administration and VX challenge for treatment, the time-course for reversible, GAL-induced inhibition of whole blood AChE was measured. Fig. 3 shows the effect of i.m. injection of 4 and 8 mg/kg GAL on whole blood AChE activity over time. Inhibition of AChE was measurable after 5 min and reached maximal inhibition after 30 min for both GAL doses. Metabolism and clearance of GAL from the blood are fairly rapid as AChE activity returned to baseline levels by 4 h. Guinea pigs receiving 4 mg/kg GAL showed a peak GAL effect (61.7 ± 4.6% of control AChE activity) at 30 min, or approximately 38% inhibition. Doubling the GAL dose (8 mg/kg) inhibited AChE by a greater amount (44.9 ± 3.4% of control) at 30 min after GAL injection, yielding approximately 55% inhibition.

A 1 min post-exposure treatment strategy was attractive for the following reasons. Based on these data, GAL is available to bind AChE within minutes after i.m. injection, and therefore free GAL is available to protect a critical fraction of AChE from irreversible VX binding. Warfighters in the field are likely to receive a treatment against nerve agent within minutes of being exposed. The 1 min post-nerve agent treatment interval is standard for ATR and 2-PAM. It is apparent that full recovery of AChE from the reversible, inhibitory effects of GAL requires approximately 4 h for completion. Thus, 4 h was selected as the duration for monitoring recovery of force tensions in GAL exposed muscle.

Relationship between GAL dose and red blood cell (RBC) AChE activity

RBC AChE activity was plotted in Fig. 4 as a function of the various GAL treatment doses. The reported AChE activities plotted represent a snapshot in time of the AChE activity at 30 min after GAL injection. The relationship is important because a range between 30 and 60% inhibition
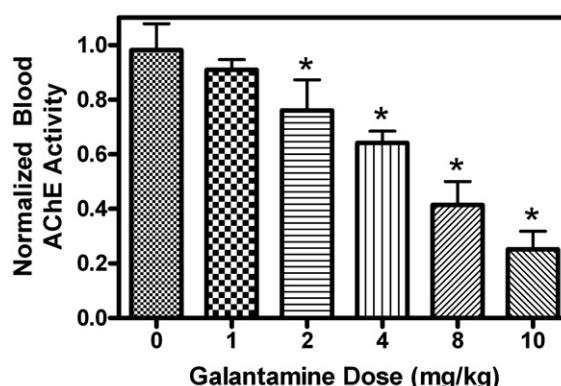

Fig. 4. Relationship between GAL dose and RBC AChE activity. A baseline AChE activity was measured from whole blood prior to i.m. saline (0 GAL) or GAL injection (1, 2, 4, 8, or 10 mg/kg). AChE activity was determined from blood collected 30 min after GAL injection. Normalized RBC AChE activity was calculated by dividing the AChE activity after 30 min by each baseline AChE activity. Normalized RBC AChE activity was plotted as a function of GAL dose. Vertical bars represent the mean ± SEM of data obtained from 4–6 animals. Reported percent of control activity is derived by multiplying the normalized activities by 100%. The symbols (*) indicate when normalized AChE activities were significantly different ($p < 0.05$) compared to control (0 GAL).
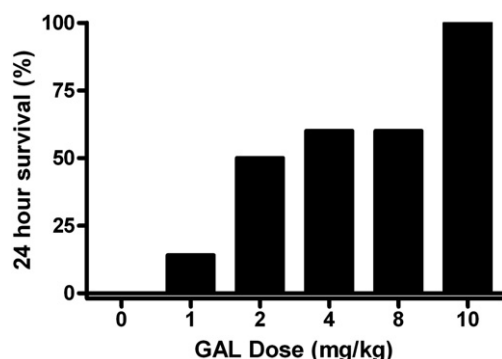
Fig. 5. Effect of GAL in the post-nerve agent exposure treatment period on VX-induced lethality. Vertical bars represent the percent survivors from 7–10 animals.

was shown to be efficacious against nerve agent for the reversible, AChE inhibitor PB (Hilmas et al., 2006). In GAL treated guinea pigs, a dose range between 2 and 8 mg/kg was shown to produce between 24% (76.1 ± 11.1% activity of control) and 59% (41.4 ± 8.7% activity of control) inhibition, respectively. All doses of GAL tested produced a statistically significant ($p < 0.05$) percent inhibition of RBC AChE activity compared to the saline (0 mg/kg) treatment group. The largest GAL dose (10 mg/kg) produced the greatest inhibition of AChE (25.2 ± 6.6% activity of control).

Survival study: GAL protection against VX-induced lethality

The effect of post-exposure treatment GAL in guinea pigs challenged with 2LD50 VX is shown in Fig. 5. Saline substituted for GAL (0 mg/kg GAL) did not protect guinea pigs; saline led to 100% lethality. In all cases, guinea pigs received 0.5 mg/kg ATR and 25 mg/kg 2-PAM, but this treatment alone was not enough to prevent death in the saline-challenged treatment group. This dose of VX was selected to yield no guinea pig survivors even when provided the nerve agent antidote ATR + 2-PAM. All GAL doses (1, 2, 4, 8, and 10 mg/kg) tested demonstrated efficacy to prevent 2LD50 induced lethality. A 10 mg/kg GAL dose protected 100% of guinea pigs from the lethal effects of 2LD50 VX. In comparison, PB (10 or 40 μg/kg), in combination with ATR + 2-PAM, resulted in 100% guinea pig lethality against 2LD50 VX when administered in the post-exposure treatment period (data not shown).

Protection of diaphragmatic function

Typical tetanic tensions displayed from various guinea pig diaphragm experiments (saline controls and groups challenged with

2LD50 VX in the absence or presence of GAL post-exposure treatment) are shown in Fig. 6. In all cases, diaphragmatic tetanic tensions were elicited by 100 Hz stimulus trains via the phrenic nerve, and the traces shown represent the first recordings after dissection and equilibration of the muscles (~20 min on average after euthanization). VX (2LD50) with saline treatment (0 GAL) was enough to produce significant fatigue and fade in the ex vivo diaphragm assay. Fade was usually evident within the first 50 ms of the stimulus train; fade is defined as the inability of the muscle to maintain tension during the time course of repetitive nerve stimulation (see second trace from the left). Recovery is also evident in these traces; although the muscle tissues were mounted immediately after the animal was euthanized, the traces were recorded 80 min on average after nerve agent exposure. In the presence of increasing concentrations of GAL, fade becomes less evident, and the diaphragm tension curve assumes the shape of the control saline-challenge group; however, peak tensions and area under the curve (AUC) were all decreased compared to control diaphragms. Over a 4-h recovery period in the tissue bath, these recordings demonstrate minimal to no fade, dose-dependent enhancement in peak tension amplitudes, and dose-dependent increases in AUC for all doses of GAL (1–10 mg/kg) tested (traces not shown). The GAL alone group was shown to have no effect on indirectly-elicited muscle tensions at these same doses (traces not shown); these traces appeared identical to the saline-challenged treatment group.

Ex vivo diaphragmatic tensions can vary in amplitude between experiments. Therefore, it was appropriate to normalize the data by dividing the AUC elicited from tetanic (50, 100, or 200 Hz) tensions by the AUC generated from twitch (0.05 Hz) tensions. There were striking dose-dependent GAL effects on tension AUC ratios generated in diaphragm muscle from guinea pigs exposed to 2LD50 VX (Fig. 7B). GAL produced dose-dependent increases in normalized tetanic/twitch AUC ratios. GAL post-exposure treatment led to a progressive enhancement of peak tetanic tension and AUC over time. At 8 mg/kg GAL doses, AUC ratios for 100 and 200 Hz were 9.8 ± 0.5 and 9.5 ± 1.4, respectively, for the first series (Fig. 7A; initial muscular paralysis) compared to 23.7 ± 3.8 and 35.4 ± 7.4, respectively, on the final 4-h recovery series (Fig. 7B; 4-h recovery after muscular paralysis).

After a 4-h recovery period in the tissue bath, AUC tetanus-twitch ratios were close to control values at the highest GAL doses (8 and 10 mg/kg) tested. AUC ratios exhibited a dose-dependent recovery at all GAL doses. Increases in the AUC tetanus/twitch ratios noted between the initial paralysis and recovery graphs reflect the greater degree of recovery of the fraction of AChE that was inhibited by the reversible AChE inhibitor (Berry and Davies, 1970; Gordon et al., 1978; French et al., 1979; Hilmas et al., 2006) GAL.
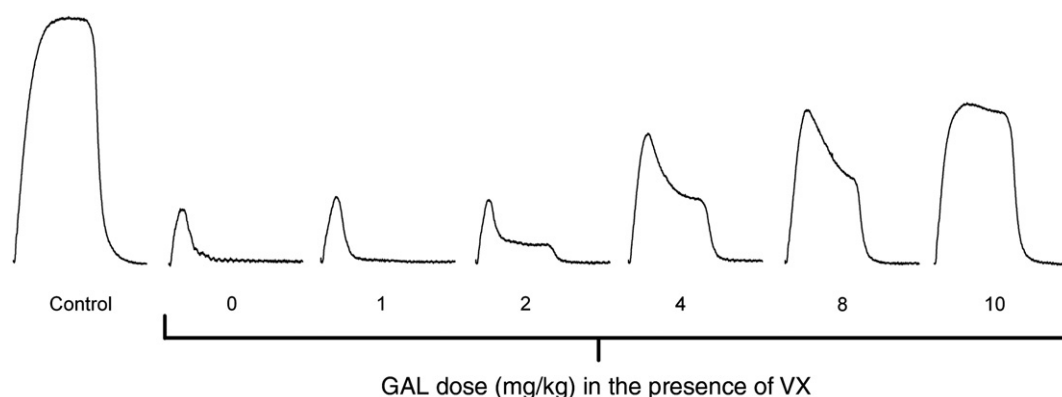

Fig. 6. Representative diaphragmatic tetanic tensions elicited by repetitive stimulation of the phrenic nerve in the absence (control) and presence of VX. The following conditions are represented: Control (saline, no VX) and VX treatment groups (0, 1, 2, 4, 8 or 10 mg/kg GAL). All guinea pigs were injected with atropine (0.5 mg/kg)/2-PAM (25 mg/kg) 1 min after VX challenge. Tetanic tensions shown are those recorded from post-exposure treatment groups immediately after mounting the ex vivo hemidiaphragm in the vertical tissue bath. All tetanic tensions shown were elicited from repetitive stimulation at 100 Hz for 200 ms.
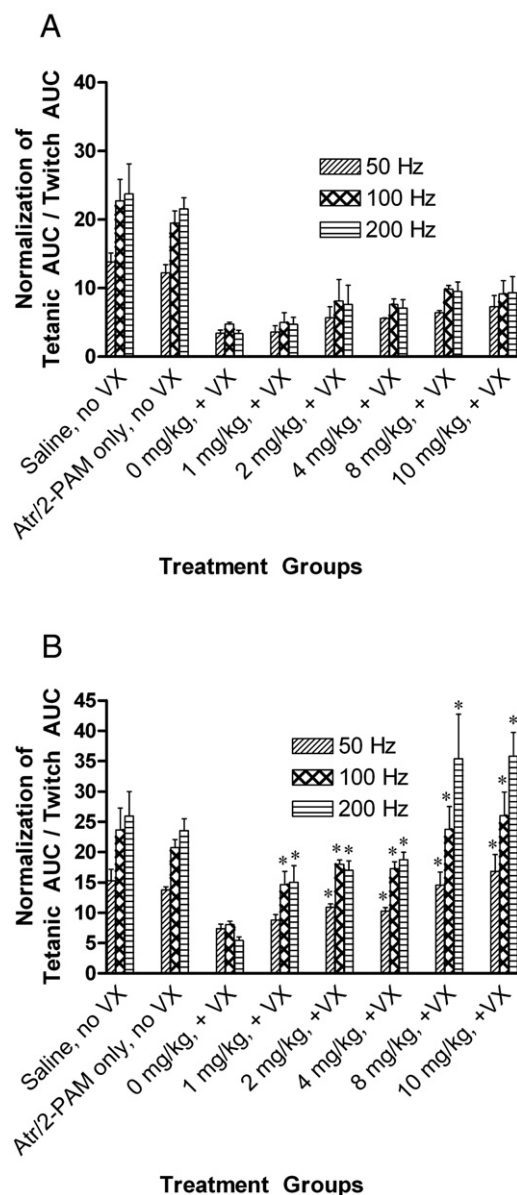
Fig. 7. Tetanic tension AUC/twitch tension AUC ratios calculated for various GAL post-exposure treatment groups in guinea pigs. (A) Graph depicts recovery of tetanic/twitch tension ratios 80 min after s.c. VX injection. The bars represent AUC ratios calculated from tensions recorded approximately 80 min after VX at 3 stimulation frequencies (50, 100, and 200 Hz). (B) Graph depicts recovery of tetanic/twitch tension ratios 320 min after s.c. VX injection. The vertical bars represent AUC ratios 4-h later compared to Graph A (or 320 min after VX). Animals in each experimental group received 2LD50 VX delivered s.c. between the shoulder blades, followed 1-min later by ATR (0.5 mg/kg) and 2-PAM (25 mg/kg), administered as a single injection i.m. Guinea pigs were injected with GAL (0, 1, 2, 4, 8 or 10 mg/kg, i.m.), 1 min after VX administration. Two extra control groups labeled 'saline only' and 'Atr/2-PAM' only were included in the study design. Vertical bars and lines represent the mean ± SEM of results from $n = 4$–9 muscles). All twitch tensions were recorded at 0.05 Hz. Tetanic tensions were recorded at 50, 100, and 200 Hz frequencies. Symbol (*) represents statistical significance $p < 0.05$ compared to 0 mg/kg GAL, + VX treatment group.

Effect of GAL on seizure onset and duration

GAL post-exposure treatment does not alter the time of seizure onset (see Fig. 8). It was hypothesized that since GAL is a reversible inhibitor of AChE, there might be a chance that it would shorten the seizure onset induced by VX. There was no statistically significant variation in the seizure onset times for all GAL doses evaluated.

GAL does affect seizure duration when administered as a post-exposure therapeutic (see Fig. 9). At 10 mg/kg, GAL produces a
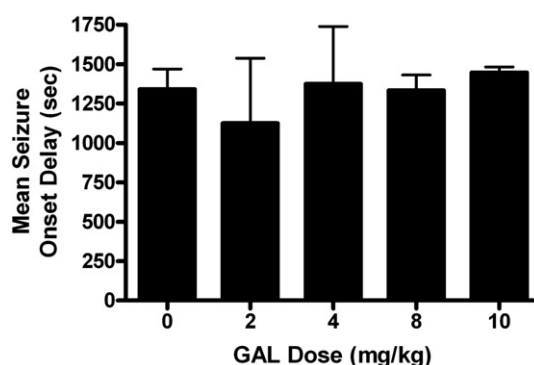

Fig. 8. Effect of GAL dose on VX-induced seizure onset. For each dose of GAL (0, 2, 4, 8, or 10), the average onset of seizures is plotted. Vertical bars and lines represent the averages ± SEM from $n = 8$–10 exposures. All animals in each experimental group received 2LD50 VX delivered s.c. between the shoulder blades, followed 1-min later by ATR (0.5 mg/kg) and 2-PAM (25 mg/kg), administered as a single injection i.m.

statistically significant decrease in seizure duration when administered as a post-exposure therapeutic against 2LD50 VX. This was the only dose able to turn off seizures within the 4-h recording period; even if seizure activity broke through, this dose tended to turn off seizures once more. The other doses of GAL were ineffective to reduce seizure duration. If seizures started, any dose less than 10 mg/kg GAL did not reduce VX-induced seizure duration to any appreciable level.

Discussion

GAL is an effective adjunct therapeutic in combination with ATR and 2-PAM antidotal therapy against lethal VX. The GAL dose range evaluated in this study offered protection from VX-induced lethality and respiratory paralysis. In the present study, GAL i.m. protected guinea pigs from 2LD50 VX-induced lethality in the presence of the muscarinic antagonist ATR (0.5 mg/kg) and the oxime 2-PAM (25 mg/kg). In the absence of GAL, the standard NA antidote cocktail (ATR + 2-PAM) was ineffective in preventing VX-induced lethality. GAL produced a dose-dependent enhancement of survival against lethal VX challenge. GAL protects the diaphragm muscle from VX-induced respiratory paralysis in a dose-dependent manner. In contrast, the reversible carbamate pretreatment PB, indicated for soman nerve agent intoxication, failed to enhance survival and protect the diaphragm when administered as a post-exposure therapeutic (10 μg/kg or 40 μg/kg) against a lethal 2LD50 VX challenge (data not shown).
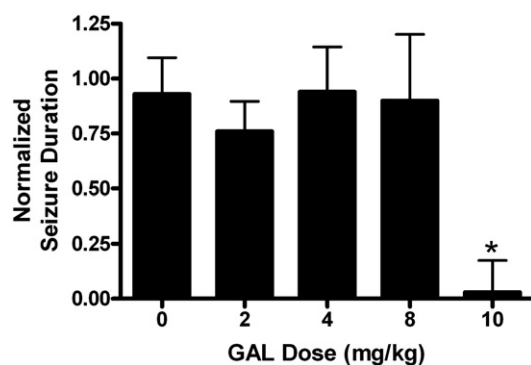

Fig. 9. Effect of GAL dose on VX-induced seizure duration. For each dose of GAL (0, 2, 4, 8, or 10 mg/kg), seizure duration was normalized such that the total seizure time was divided by the total recording period (typically 4-h). Normalized seizure duration was plotted as a function of GAL, administered as a post-exposure therapeutic. Vertical bars and lines represent the averages ± SEM from $n = 8$–10 exposures. The symbol (*) indicates that normalized seizure duration is significantly different from control (0 GAL).

Electrophysiologically, GAL did demonstrate anticonvulsant properties. GAL did not attenuate seizure activity once it had started (data not shown); GAL also did not alter the time to seizure onset (Fig. 8). A shortening of the seizure onset time was expected considering the fact that both GAL and VX share a common mechanism of action, inhibition of AChE. Additionally, GAL was expected to reduce seizure duration as it unbinds from AChE over time. The highest dose of GAL (10 mg/kg) reduced seizure duration as anticipated; the lower doses, which were effective in prolonging survival against lethal VX challenge, were not effective in reducing seizure duration.

The present study was consistent with the Albuquerque et al. (2006) study, both showing that GAL was an effective adjunct with ATR and 2-PAM to enhance survival from lethal nerve agent challenge. However, there were several important differences between the two studies. This study used VX as opposed to sarin or soman. Additionally, our challenge dose was fixed at 2LD50 VX, compared to 1.5 and 2 LD50 in the Albuquerque study. The present study fixed the dose of ATR at 0.5 mg/kg to reflect the dose a warfighter might receive in the field for treatment of severe NA intoxication. The Albuquerque study fixed ATR at 10 mg/kg as treatment for 2LD50 soman or varied the ATR dose (4–16 mg/kg) against a 1.5LD50 soman challenge.

It is known that ATR demonstrates anticonvulsant properties at high doses (Green et al., 1977). Large doses of ATR can counteract the convulsive effects of significant NA poisoning. Pazdernik et al. (1986) showed that high dose ATR (10 mg/kg) was found, similar to diazepam, to decrease cerebral glucose utilization and therefore reduce brain damage. It was suggested that the effectiveness of high ATR against NA intoxication was due to more than muscarinic blockade. The reported anticonvulsant ED50s of atropine for VX and soman are 4.1 mg/kg and 12.2 mg/kg, respectively (Shih and McDonough, 2000). Therefore, high ATR (10 mg/kg) is expected to provide anticonvulsant benefit. In the present study, it was surprising that GAL enhanced survival in guinea pigs against 2LD50 VX at 0.5 mg/kg ATR, below the threshold for anticonvulsant action.

An interesting finding from our seizure data was the change in correlation between seizure activity and lethality in the absence and presence of GAL. In guinea pigs, the correlation between developing a seizure after 2LD50 VX challenge and animal death was very high ($r = 0.876$). However, in the presence of effective GAL (2–10 mg/kg), the correlation between seizures and death was reduced ($r < 0.385$). Therefore, GAL treatment promotes guinea pig survival despite the presence of NA-induced seizure activity in the animal. This would highlight the importance of peripheral protective mechanisms by GAL, including diaphragmatic protection.

Comparing the IC50s for GAL inhibition of AChE and butyrylcholinesterase (BuChE), GAL has an approximate 53-fold greater potency for AChE than BuChE (Lilienfeld, 2002; Thomsen et al., 1990; Thomsen and Kewitz, 1990; Thomsen et al., 1991). It is suggested that the effectiveness of GAL could be related to its preference for AChE and therefore preservation of remaining plasma BuChE to scavenge nerve agent (Albuquerque et al., 2006). Although this scavenging capacity of plasma BuChE is present, the protective effect of GAL against VX in our study was demonstrated to be related to peripheral protection of the diaphragm from respiratory paralysis, a major cause of death from OP nerve agents. For each GAL dose demonstrating enhancement of survival from lethal VX challenge, the diaphragm showed significant recovery from paralysis. Both GAL-related effects were dose-dependent in the same range.

Acknowledgments

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